Aspartame Attenuates 2, 4-Dinitrofluorobenzene-Induced Atopic Dermatitis–Like Clinical Symptoms in NC/Nga Mice

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Atopic dermatitis (AD) is a common multifactorial chronic skin disease that has a multiple and complex pathogenesis. AD is gradually increasing in prevalence globally. In NC/Nga mice, repetitive applications of 2, 4-dinitrofluorobenzene (DNFB) evoke AD–like clinical symptoms similar to human AD. Aspartame (*N*-L-α-aspartyl-L-phenylalanine 1-methyl ester) is a methyl ester of a dipeptide, which is used as an artificial non-nutritive sweetener. Aspartame has analgesic and anti-inflammatory functions that are similar to the function of nonsteroidal anti-inflammatory drugs such as aspirin. We investigated whether aspartame can relieve AD–like clinical symptoms induced by DNFB treatment in NC/Nga mice. Sucrose did not relieve AD–like symptoms, whereas aspartame at doses of 0.5 μg kg^{-1} and 0.5 mg kg^{-1} inhibited ear swelling and relieved AD–like clinical symptoms. Aspartame inhibited infiltration of inflammatory cells including eosinophils, mast cells, and $CD4^+$ T cells, and suppressed the expression of cytokines including IL-4 and IFN-γ, and total serum IgE levels. Aspartame may have therapeutic value in the treatment of AD.

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INTRODUCTION

Atopic dermatitis (AD) is a serious and chronically relapsing inflammatory skin disorder involving severe pruritus, dryness, and clinical manifestations such as erythematous, edema, and lichenification in skin lesions (Leung and Bieber, 2003). These clinical symptoms correlate with IgE–mediated sensitization, increased transepidermal water loss, and cutaneous hypersensitivity that leads to disruption of the skin barrier (Gittler *et al.*, 2013). The estimated number of AD patients includes up to 5% of adults and 18% of children worldwide, and the prevalence has been increasing steadily (Shaw *et al.*, 2011). AD has a complicated pathogenesis involving genetic, immunologic, and environmental factors, which hinders efficacious treatment (Leung, 2000). Although long-term application of steroids can produce side effects that include skin thinning and atrophy, topical corticosteroid therapy is a common and effective treatment for AD (Rudikoff and Lebwohl, 1998). There is a need for new kinds of treatment for AD.

Aspartame (*N*-L-α-aspartyl-L-phenylalanine 1-methyl ester) is a methyl ester of a dipeptide used as an artificial non-nutritive sweetener. It is marketed as NutraSweet, Canderel, Sanecta, and Equal (Baines, 1985; Magnuson *et al.*, 2007). Aspartame is the most widely used synthetic sweetener in various foods, drugs, and beverages reflecting its inexpensive cost, stability under dry conditions, reduced caloric intake, marked (150 to 200 times) increased sweetness compared with traditional sugar, and the absence of proven side effects (Magnuson *et al.*, 2007). Because of these properties, aspartame has popularly been used to reduce or maintain body weight (Pandurangan *et al.*, 2014). Aspartame also significantly reduces lipid accumulation and expression of peroxisome proliferator–activated receptor γ, fatty acid–binding protein 4, CCAAT/enhancer-binding protein α, and sterol regulatory element–binding protein 1 during 3T3-L1 differentiation without significant toxic effects (Pandurangan *et al.*, 2014).

Aspartame has analgesic functions that are similar to the function of nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin. These functions include increased motor activity and attenuated mechanical hyperalgesia in a carrageenan-elicited monoarthritis murine model (LaBuda and Fuchs, 2001). Aspartame also reportedly reduces lipopolysaccharide-stimulated IL-6, which is a recognized biomarker of the inflammatory response, and it slightly increases the anti-inflammatory cytokine IL-10 in human whole-blood cultures (Rahiman and Pool, 2014). Although there is no direct evidence for the anti-inflammatory functions of aspartame, it may involve the inhibition of prostaglandin H_2 synthesis and cyclooxygenases. Computer-assisted virtual modeling has shown that aspartame is a suitable ligand for

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Abbreviations: AD, atopic dermatitis; Aspartame, N-L-α-aspartyl-L-phenylalanine 1-methyl ester; DNFB, 2, 4-dinitrofluorobenzene; DNP, dinitrophenyl

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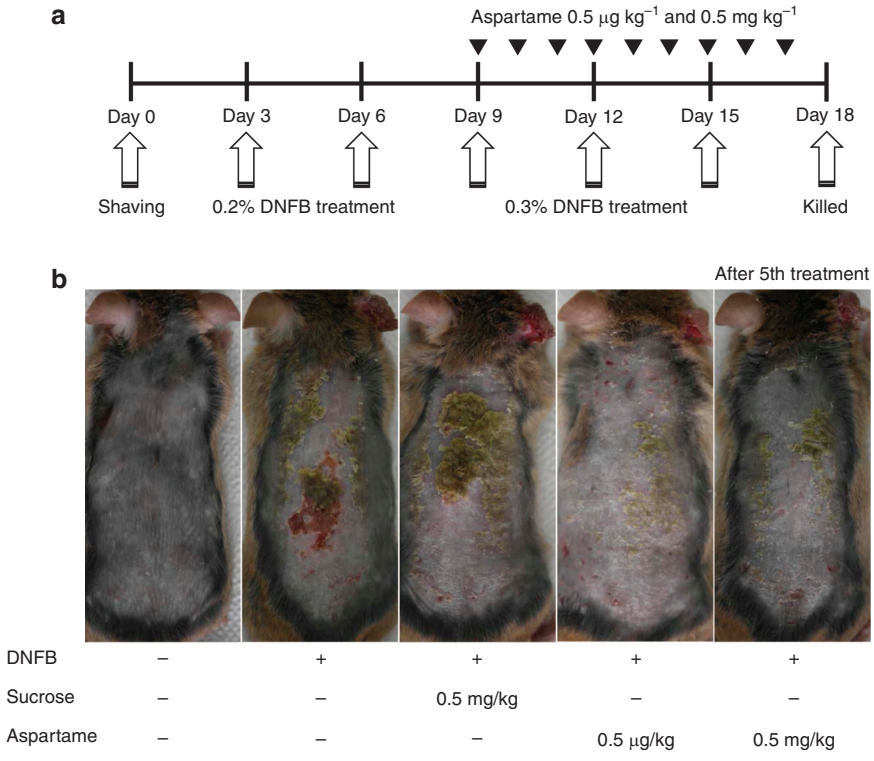
human Bence–Jones dimer, which represents an Ig light chain (Edmundson and Manion, 1998). This complex of aspartame and the Bence–Jones dimer have been closely matched to the active site of the cyclooxygenase portion of prostaglandin H_2 synthase-1 and they interact with IgM rheumatoid factors (Edmundson and Manion, 1998; Rahiman and Pool, 2014). In patients with rheumatoid arthritis, osteoarthritis, treatment with aspartame increased bleeding time, relieved pain, and improved performance including walking, climbing, and grip strength (Edmundson and Manion, 1998).

We hypothesized that the analgesic effect of aspartame may attenuate AD symptoms. Therefore, we investigated the therapeutic effects of aspartame on AD–like skin lesions.

RESULTS

Aspartame ameliorates DNFB-induced AD–like clinical manifestations

Repetitive application of 2, 4-dinitrofluorobenzene (DNFB) induced AD–like clinical symptoms in NC/Nga mice (Jin *et al.*, 2009). Aspartame ameliorates mechanical hyperalgesia in a carrageenan-induced monoarthritis model (LaBuda and Fuchs, 2001). Thus, we investigated the therapeutic effects of aspartame on DNFB-induced AD–like symptoms and responses. Mice were randomly divided into five groups. Mice were sensitized by 0.2% DNFB on days 3 and 6, and then they were further challenged with 0.3% DNFB on days 9, 12, and 15 (Figure 1a). The effects of aspartame treatment were then macroscopically assessed at



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Groups	SCORAD	No. of mice
DNFB (–)	0 ± 0.00	5
DNFB	9.83 ± 1.09	5
DNFB (+) SUC 0.5 mg/kg	8.33 ± 1.08 *	5
DNFB (+) ASP 0.5 μg/kg	6.00 ± 1.29 ***	5
DNFB (+) ASP 0.5 mg/kg	5.33 ± 1.25 ***	5

Figure 1. Aspartame (ASP, *N*-L-α-aspartyl-L-phenylalanine 1-methyl ester) relieves 2, 4-dinitrofluorobenzene (DNFB)-induced atopic dermatitis (AD)–like symptoms. (**a**) AD–like lesions were induced by repetitive painting of 0.2% DNFB (acetone:olive oil, 3:1) on the dorsal skin of NC/Nga mice once daily on days 3 and 6, with further challenge with 0.3% DNFB on days 9, 12, and 15. (**b**) Representative dorsal skin photographs captured after the fifth treatment depict AD–like skin lesions with and without Aspartame ($n = 5$ in each group). (**c**) AD severity was macroscopically evaluated by SCORAD (SCORing Atopic Dermatitis). Overall dermatitis score was determined from the sum of all individual scores. Data are presented as mean ± SEM of five determinations. $^*P < 0.05$, $^{***}P < 0.001$ vs. DNFB (+) groups. SUC, sucrose.

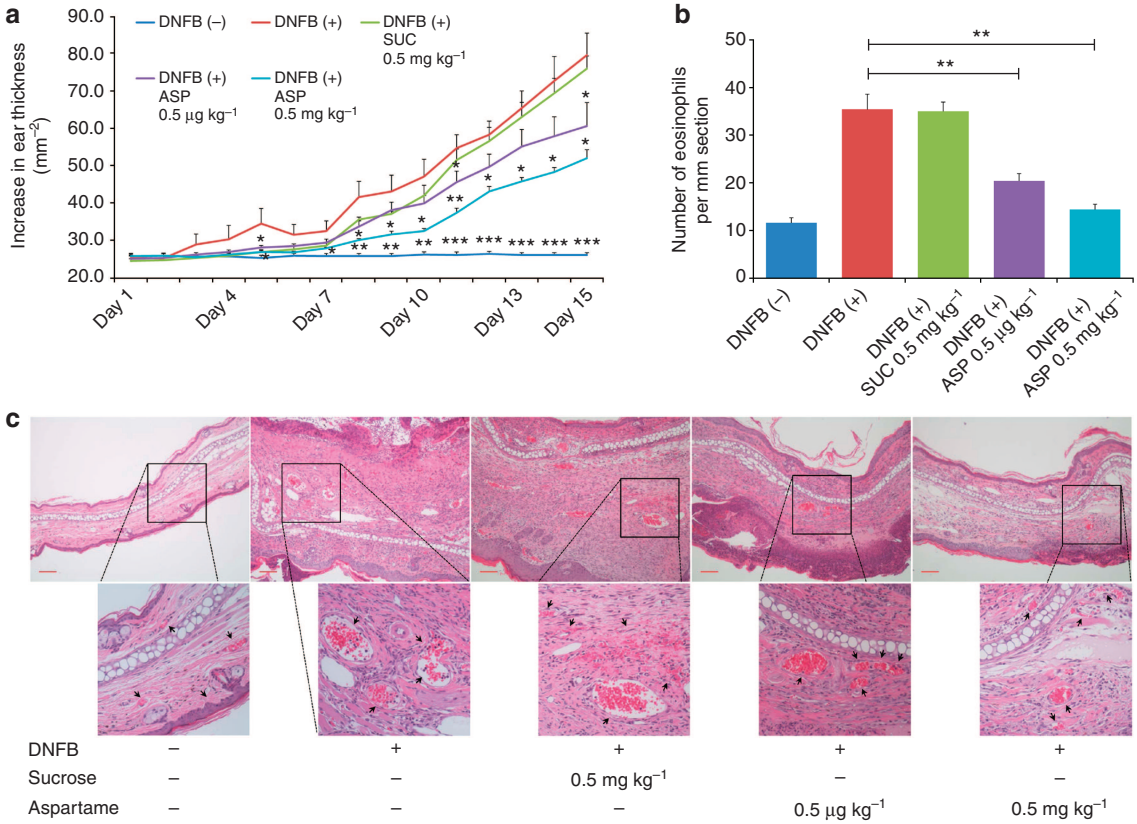

Figure 2. Aspartame (ASP, *N*-L-α-aspartyl-L-phenylalanine 1-methyl ester) reduces increasing ear swelling and epidermal thickness. (**a**) Ear swelling was measured daily with a thickness gauge. Aspartame and sucrose (SUC) were orally administered at doses of 0.5 μg kg^{-1} or 0.5 mg kg^{-1}. Five mice were placed into each of five groups: 2, 4-dinitrofluorobenzene (DNFB) (−; acetone:olive oil, 3:1); DNFB (+; acetone:olive oil, 3:1 with 0.2–3% DNFB); DNFB with sucrose, 0.5 mg kg^{-1}; DNFB with aspartame, 0.5 μg kg^{-1}; and DNFB with aspartame, 0.5 mg kg^{-1}. (**b**) Skin sections were hematoxylin and eosin–stained ($n = 10$). (**c**) Bar = 100 μm. Data are presented as mean ± SEM of five determinations. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. DNFB (+) groups.

the site of DNFB-induced AD–like skin lesions. The acceptable daily intake level of aspartame in humans is 40 and 50 mg kg^{-1} of body weight according to the European Food Safety Authority and US Food and Drug Administration, respectively (Magnuson *et al.*, 2007). At present, continual repetitive application of DNFB-induced AD–like skin lesions involving severe clinical manifestations including erythema, edema, oozing, excoriation, crust, and lichenification in NC/Nga mice. Daily oral administration of 0.5 mg kg^{-1} sucrose was not therapeutic, whereas oral administration of 0.5 μg kg^{-1} and 0.5 mg /kg^{-1} aspartame relieved AD–like skin lesions compared with the DNFB-treated group (Figure 1b). DNFB-evoked ear swelling was closely correlated with relapsing edema and oozing, and this response was significantly suppressed by aspartame in a concentration-dependent manner (Figures 1 and 2a).

Aspartame inhibits infiltration of inflammatory cells to AD–like skin lesions

Repetitive applications of DNFB markedly disturb the epidermal barrier and induce adhesion molecules. Levels of various immune mediators are increased by DNFB treatment, which induce the cytokine cascade and increase epidermal leukocyte density that leads to leukocyte rolling and infiltration of inflammatory cells (Proksch and Brasch, 1997; Deane and Hickey, 2009). Increasing numbers of FcεRI (high affinity Immunoglobulin E receptor)-expressing eosinophils and mast cells have an important role in developing chronic AD (Abboud *et al.*, 2009). In AD, infiltrated eosinophils secrete lipid mediators, toxic granule proteins, cytokines, and enzymes including eosinophil peroxidase, and major basic protein that induces tissue damage and inflammation in skin lesions (Wakita *et al.*, 1994). Therefore, we examined the effect of aspartame on dermal infiltration of eosinophils in AD–like skin lesions in NC/Nga mice. DNFB induced enormous infiltration of eosinophils and dermal thickness in ear and dorsal skin lesions (Figures 2 and 3). Sucrose at a dose of 0.5 mg kg^{-1} did not suppress infiltration of inflammatory cells and hyperkeratosis in AD–like skin lesions. Both aspartame concentrations inhibited infiltration of eosinophils and suppressed epidermal thickening. Activation of mast cells through the FcεRI receptor by allergen-sensitized IgE leads to the release of various granular mediators, cytokines, and chemokines including prostaglandins, leukotrienes, platelet-activating factor, IL-13, and eotaxin, which are associated with the development of AD (Amon *et al.*, 1994). Thus, we

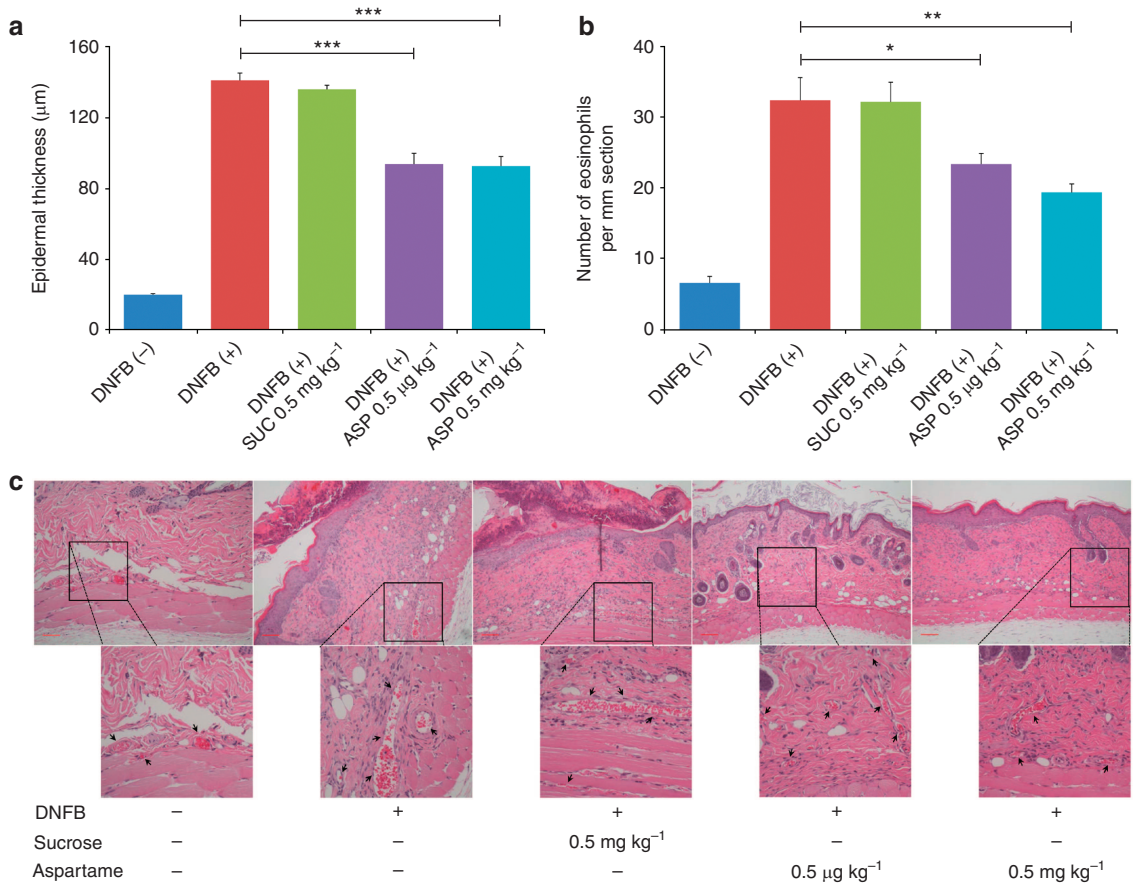

Figure 3. Aspartame (ASP, *N*-L-α-aspartyl-L-phenylalanine 1-methyl ester) inhibits infiltration of eosinophils in atopic dermatitis (AD)–like skin lesions. (**a**) Skin sections were hematoxylin and eosin (H&E) stained ($n = 10$). (**b**) Eosinophils in H&E–stained sections were counted under a microscope, and the numbers of infiltrated eosinophils were expressed as average total counts in three fields of 100 μm^2. (**c**) Bar = 100 μm. The number of cells is expressed as the mean ± SEM of five sections. Arrows indicate eosinophils. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. DNFB (+) groups. SUC, sucrose.

examined the suppressive effect of aspartame on degranulation and infiltration of mast cells in DNFB-induced AD–like skin lesions using toluidine blue staining. Both concentrations of aspartame reduced mast cell infiltration and degranulation, whereas 0.5 mg kg^{-1} sucrose did not (Figure 4). T lymphocytes have important roles for the development of AD—that is, as a critical source of Th_2 cytokines, such as IL-4, IL-5, and IL-13, which are important to IgE synthesis and eosinophil accumulation in AD–like skin lesions (Novak *et al.*, 2004). We examined whether aspartame inhibited infiltration of T lymphocytes in AD–like skin lesions using immunofluorescence. Sucrose did not suppress infiltration of T lymphocytes, whereas aspartame inhibited infiltration of $CD4^+$ T cell numbers in dorsal skin lesions in NC/Nga mice (Figure 5).

Aspartame suppresses inflammatory cytokine synthesis by activated $CD4^+$ T cells of DNFB-induced AD–like skin lesions

AD is characterized by two distinct phases, the chronic and acute phases, resulting in either T helper type 1 (Th1) or T helper type 2 (Th2) dominant inflammation. Th1 dominant inflammation is mainly affected by Th1 cells and their production of IL-2 and IFN-γ, whereas IL-4, IL-5, and IL-13 are produced by Th2 cells that affect Th2 dominant inflammation (Leung, 2000). IL-2 and IFN-γ contribute to hyperkeratosis and dermal thickening that leads to the chronic phase of AD. Th2 cytokines induce the synthesis and secretion of IgE and accumulation of inflammatory cells in AD–like skin lesions, which also lead to the development of the chronic phase of AD. Therefore, we investigated whether aspartame affects the Th1 or Th2 responses of $CD4^+$ T cells in the AD–like NC/Nga mouse model. Aspartame inhibited the infiltration of $CD4^+$ T cells in DNFB-induced AD–like dorsal skin lesions (Figure 5). Furthermore, production of IL-4 and IFN-γ decreased in mice treated with either concentration of aspartame compared with that in DNFB-treated mice and 0.5 mg kg^{-1} sucrose-treated mice (Figure 6a and b).

Aspartame suppresses DNFB-induced serum IgE synthesis

In allergic inflammation, IgE is synthesized by B cells and induces the activation of mast cells through FcεRI, leading to the secretion of Th2 cytokines and histamine (Kim *et al.*, 2012). Because abundant synthesis of total serum IgE is a hallmark of AD (Leung, 2000), we prepared serum samples to assess total serum IgE and dinitrophenyl (DNP)-specific IgE levels using an ELISA. Consistent with other studies (Yamaguchi *et al.*,

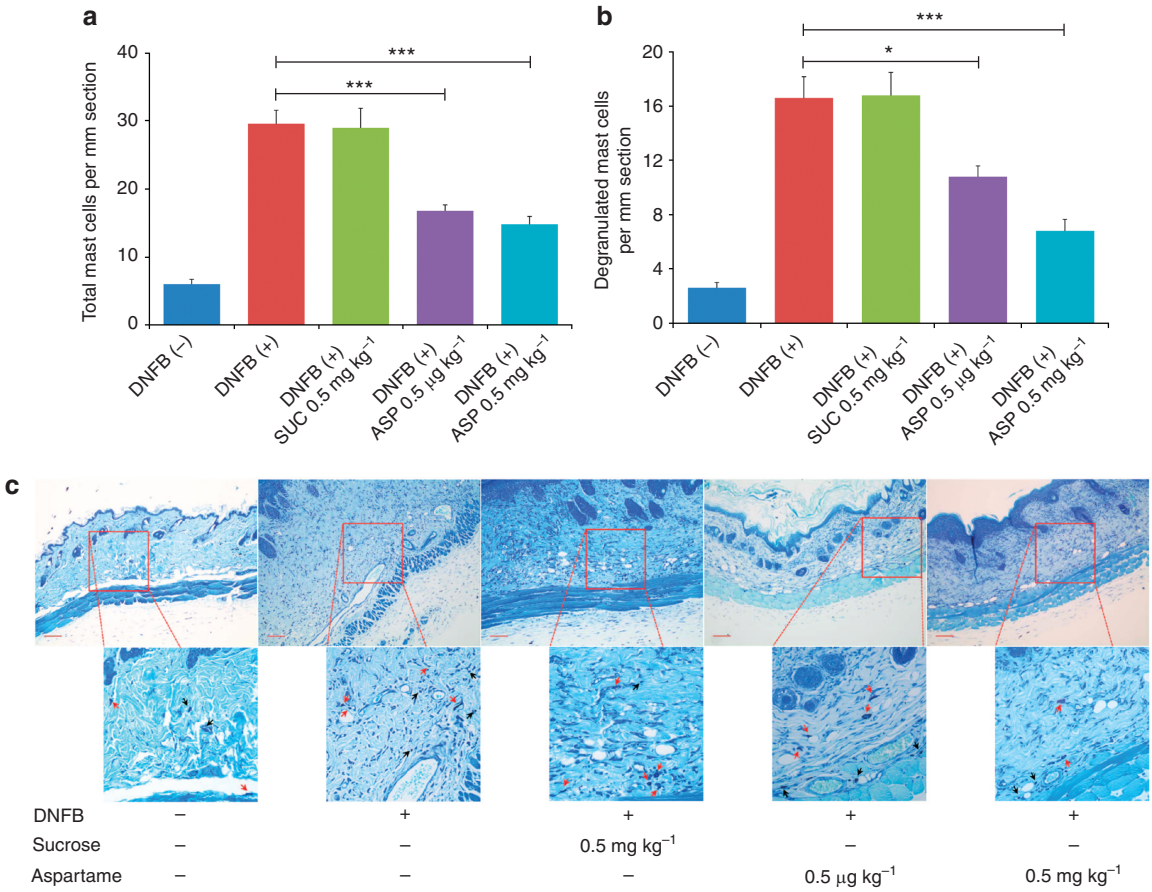

Figure 4. Aspartame (ASP, *N*-L-α-aspartyl-L-phenylalanine 1-methyl ester) suppresses degranulation and infiltration of mast cells in atopic dermatitis (AD)–like skin lesions. (**a**) 2, 4-Dinitrofluorobenzene (DNFB) (–), DNFB (+), DNFB+sucrose (SUC) (0.5 mg kg^{-1}), DNFB+aspartame (0.5 μg kg^{-1}), and DNFB+aspartame (0.5 mg kg^{-1}) treated. Mast cells in toluidine blue–stained sections were counted under a microscope. (**b**) Degranulated mast cells were expressed as average total counts in three fields of 100 μm^2 ($n = 5$ sections each). (**c**) Bar = 100 μm; the number of cells is expressed as the mean ± SEM of five sections. Both black and red arrows indicate mast cells and degranulated mast cells. $^*P < 0.05$, $^{***}P < 0.001$ vs. DNFB (+) groups.

1999), increased production of IL-4 and the infiltration and degranulation of mast cells were associated with IgE production, and both of these effects were suppressed by both concentrations of aspartame (Figures 4 and 6a). Oral administration of either concentrations of aspartame inhibited total serum IgE and DNP-specific IgE levels as compared with DNFB alone and sucrose-treated groups in the AD–like NC/Nga models (Figure 6c and d).

DISCUSSION

AD is a common chronic inflammatory skin disorder characterized by relapsing clinical manifestations including edema, erythema, scaling, and pruritus that severely impairs the quality of life of patients and family members (Galli *et al.*, 2003). Although topical steroid therapy is the most effective remedy for AD, continual repetitive application of steroids leads to side effects that include skin thinning and atrophy (Del Rosso and Friedlander, 2005). Therefore, a combination therapy including emollient, antimicrobial, and anti-inflammatory approaches is currently the preferred treatment goal for AD patients (Wollenberg and Schnopp, 2010).

Aspartame (*N*-L-α-aspartyl-L-phenylalanine 1-methyl ester) is the most widely used artificial non-nutritive sweetener in various beverages, foods, and drugs. Aspartame is 150 to 200 times sweeter than sucrose without proven side effects (Magnuson *et al.*, 2007). It is metabolized to phenylalanine, aspartic acid, and some harmful agents including methanol or formaldehyde (Bell and Labuza, 1991). However, methanol is metabolized in the liver to formaldehyde, which is rapidly metabolized to formic acid with a half-life of only to 2 min, because formaldehyde does not accumulate in the body (Barceloux *et al.*, 2002).

Aspartame has not shown harmful effects *in vivo* in doses up to 1600 mg kg^{-1} body weight per day on chromosomes of bone marrow or spermatogonial cells in rats (Bowles, 1970). Another study demonstrated that aspartame at concentrations of 25, 50, and 100 mg kg^{-1} has analgesic and anti-inflammatory effects in carrageenan-induced rat model of monoarthritis (LaBuda and Fuchs, 2001). Treatment with 10 μg ml^{-1} aspartame was reported to inhibit lipopolysaccharide-stimulated IL-6, whereas production of IL-10 was slightly increased in human whole-blood cultures (Rahiman

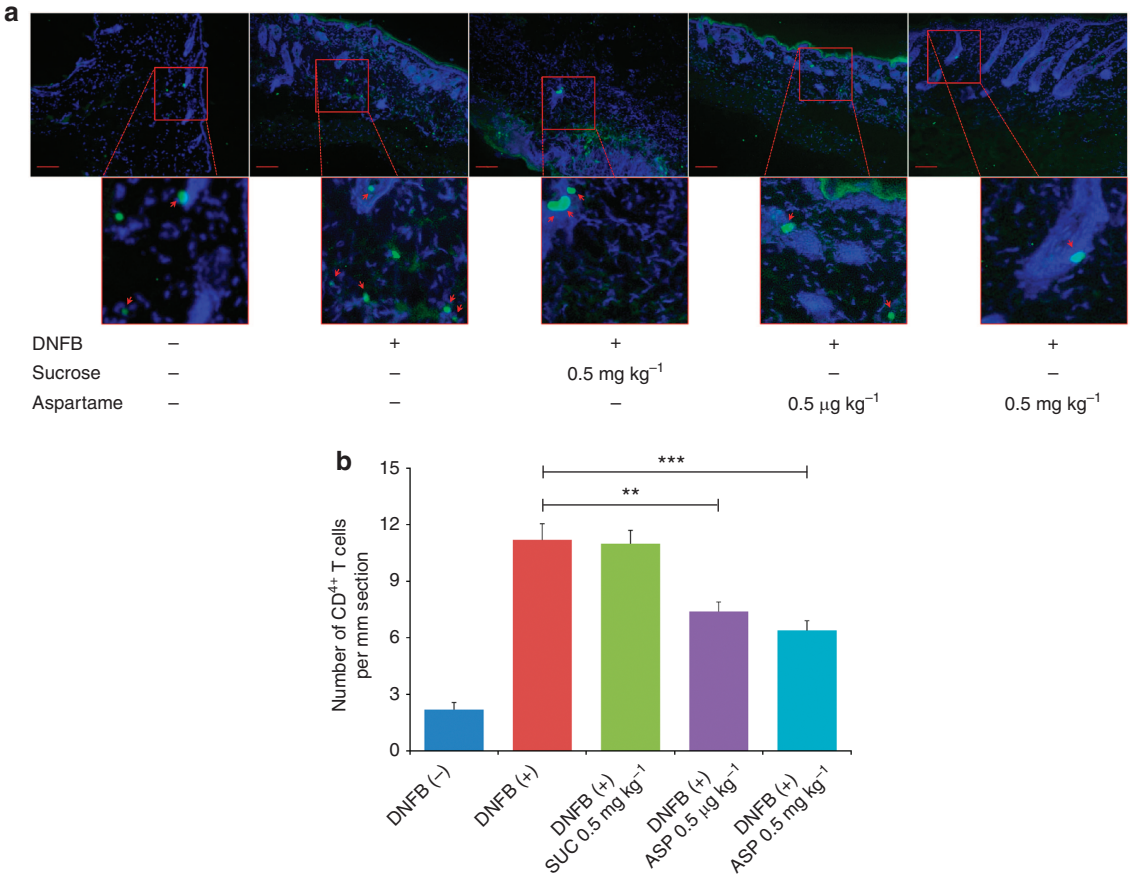

Figure 5. Aspartame (ASP, *N*-L-α-aspartyl-L-phenylalanine 1-methyl ester) inhibits infiltration of CD4$^+$ T cells in atopic dermatitis (AD)–like skin lesions. NC/Nga mice were treated with 0.2% 2, 4-dinitrofluorobenzene (DNFB) (acetone:olive oil, 3:1) on shaved back skin on days 3 and 6, and further challenged with 0.3% DNFB on days 9, 12, and 15. Aspartame was orally administered daily from the day of first DNFB challenge (day 9). On day 18, immunofluoroscence staining was performed with the cryosections of dorsal skin samples with Alexa Fluor 488–conjugated goat anti-rat antibody. Representative picture showing activation of CD4$^+$ T cells. (**a**) After DNFB treatment, which was inhibited by aspartame. (**b**) Merged CD4$^+$ T cells were counted. The number of cells is expressed as the mean ± SEM of five sections. Arrows indicate merged cells. $^{**}P<0.01$, $^{***}P<0.001$ vs. DNFB (+) groups. Bar = 100 μm. SUC, sucrose.

and Pool, 2014). Aspartame interference with the formation of complexes between normal human IgG Fc and IgM-type rheumatoid factor was described (Ramsland *et al.*, 1999). This decreasing rheumatoid factor activity leads to relived pain and improved performance involving walking and grip strength in patients with osteoarthritis (Edmundson and Manion, 1998).

These observations collectively indicate the potential value of aspartame for the treatment of AD. We investigated the effect of aspartame on the severity of AD–like clinical symptoms in DNFB-induced AD–like lesions. Treatment of mice with 0.5 μg kg^{-1} and 0.5 mg kg^{-1} aspartame significantly relieved AD–like clinical manifestations including erythema, edema, excoriation, and hyperkeratosis compared with sucrose- and DNFB-treated groups (Figure 1b and c). Furthermore, DNFB-induced ear swelling was partially inhibited by aspartame (Figure 2).

Repetitive application of DNFB disturbs the physical barrier of the skin and induces the expression of various adhesion molecules, such as vascular cell adhesion molecule and intracellular adhesion molecule, on the surface of inflammatory cells including eosinophils, mast cells, and T lymphocytes (Deane and Hickey, 2009). The secretion of various immune mediators induces the cytokine cascade and promotes accumulation of collagen, which preludes dermal thickening and increasing tissue damage (Barker *et al.*, 1991; Proksch and Brasch, 1997; Purwar *et al.*, 2008). Continual treatment of DNFB induced increased epidermal thickness and infiltration of eosinophils in ear and dorsal skin (Figures 2 and 3). Treatment with 0.5 mg kg^{-1} sucrose did not produce inhibition, whereas 0.5 μg kg^{-1} and 0.5 mg kg^{-1} aspartame reduced the increased epidermal thickness and infiltration of eosinophils.

Allergen-sensitized IgE activates mast cells, leading to the development of Th2 cells and secretion of cytokines, chemokines, prostaglandins, and platelet-activating factor, which in turn induce the development of AD–like skin lesions (Harvima *et al.*, 1994). Aspartame (0.5 μg kg^{-1} and 0.5 mg kg^{-1}) inhibited mast cell degranulation and infiltrating mast cells, whereas sucrose did not (Figure 4). Degranulation and infiltration of activated mast cells is related to either the production of inflammatory cytokines by activated T lymphocytes or increasing IgE synthesis. To investigate

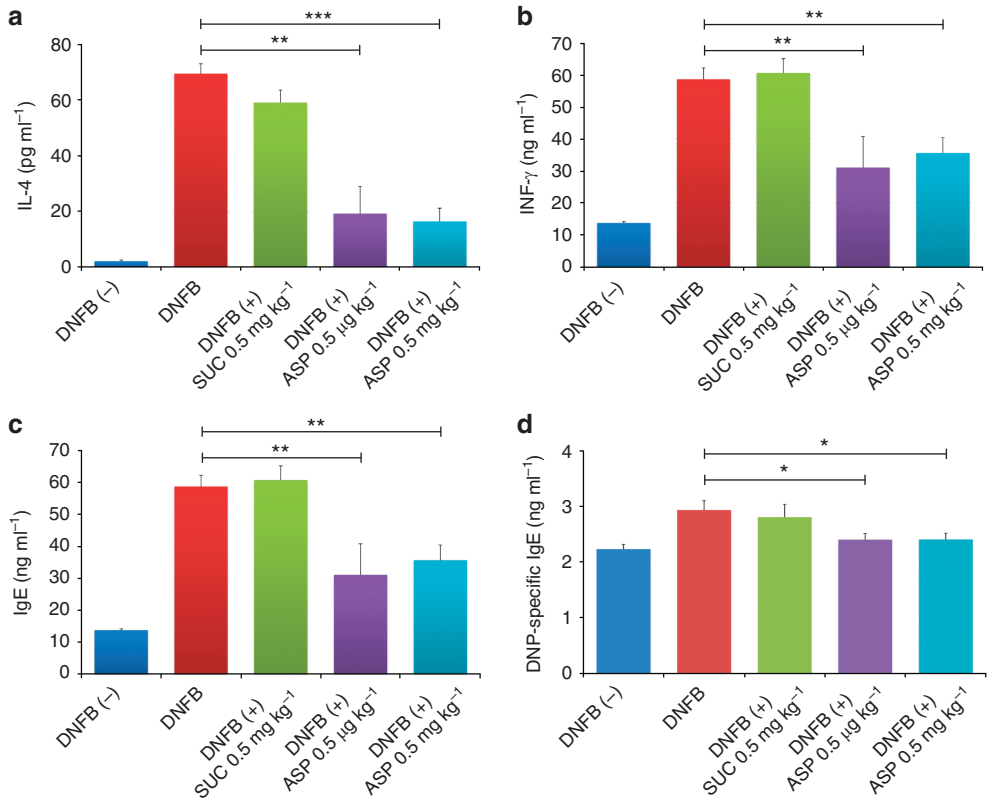

Figure 6. Aspartame (ASP, *N*-L-α-aspartyl-L-phenylalanine 1-methyl ester) decreases cytokine production of activated CD4$^+$ T cells and reduces total serum IgE levels. (**a**) After the mice were killed on day 18, lymph nodes draining the site of injection were excised, then evaluated of weights, and CD4$^+$ T cells were purified. (**b**) Purified CD4$^+$ T cells (1×10^6 cells per ml) were stimulated with anti-CD3 antibody and anti-mouse CD28 antibody for 72 h. Production of IL-4 and IFN-γ after T cell activation was quantified by ELISA. (**c**) Total IgE and (**d**) dinitrophenyl (DNP)-specific IgE in the indicated groups were measured 24 h after final administration. 2, 4-Dinitrofluorobenzene (DNFB) (−), DNFB (+), DNFB+sucrose (SUC) (0.5 mg kg^{-1}), DNFB+aspartame (0.5 μg kg^{-1}), and DNFB+aspartame (0.5 mg kg^{-1}) treated. Results are based on triplicate experiments. Data are mean ± SEM ($n = 5$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. DNFB (+) groups.

whether aspartame suppressed infiltration of T lymphocytes in AD–like skin lesions, immunofluorescence examination was carried out on dorsal skin lesions. Oral administration of 0.5 μg kg^{-1} and 0.5 mg kg^{-1} aspartame inhibited the number of infiltrating CD4$^+$ T cells (Figure 5). In allergic inflammation, Langerhans cell primed allergen specific naive T cells in draining lymph nodes develop into proallergic cells and home to the skin lesions, which is followed by the production of inflammatory cytokines (Dubrac *et al.*, 2010). To investigate whether aspartame regulates the cytokine production of CD4$^+$ T cells in dermal skin lesions, we performed ELISA. Continual treatment of DNFB markedly induced the production of IL-4 and IFN-γ by activated CD4$^+$ T cells. However, 0.5 μg kg^{-1} and 0.5 mg kg^{-1} aspartame significantly suppressed both IL-4 and IFN-γ production (Figure 6a and b). Whether aspartame inhibited cytokine production by activated CD4$^+$ T cells was not examined. However, suppressed infiltration of T lymphocytes might be correlated with decreasing cytokine production.

Th2 cytokines including IL-4, IL-5, and IL-13 produced by activated T lymphocytes are pivotal in IgE synthesis and eosinophil infiltration in AD–like skin lesions. In particular, IL-4 is critical to the rearrangement of variable diversity by joining to Cγ$_4$ or Cε in B cells (Bacharier and Geha, 2000). Total serum IgE levels and DNP-specific IgE levels were increased by DNFB in NC/Nga mice (Figure 6c and d). Treatment with 0.5 mg kg^{-1} sucrose did not reduce IgE synthesis, whereas mice treated with either aspartame concentration displayed significantly reduced production of total serum IgE and DNP-specific IgE in a concentration-dependent manner (Figure 6c and d).

Molecular mechanisms for the therapeutic effects of aspartame in AD–like models have not been fully identified. The present study suggests that aspartame may be used as a potential therapeutic compound for AD based on its analgesic functions and suppression of inflammation in AD–like NC/Nga mice. Further studies are necessary to investigate the mechanisms whereby aspartame regulates inflammatory mediator release that correlates with analgesic functions observed in the NC/Nga AD–like model.

MATERIALS AND METHODS

Animals

Seven-week-old NC/Nga male mice (19–22 g) were purchased from Japan SLC (Shizuoka, Japan) and maintained under specific pathogen-free conditions. The mice were housed in an air-conditioned animal room at a temperature of 25 ± 1 °C and a relative

humidity of 40 ± 5%, and they were fed a distilled water and laboratory diet. Animal treatment and maintenance complied with the Principles of Laboratory Animal Care (NIH Publication No. 85-23, revised in 1985) and with the guidelines issued by the ethics committee for animal welfare at Kyung Hee University (KHUASP (SE)-15-021). All procedures were conducted in accordance with the United States National Institute of Health guidelines.

Drug treatment

Mice were randomly assigned to five treatment groups: untreated (control) group, DNFB-treated group, DNFB with 0.5 mg kg^{-1} sucrose, DNFB with 0.5 μg kg^{-1} aspartame, and DNFB with 0.5 mg kg^{-1} aspartame ($n = 5$ per group). Aspartame and sucrose were dissolved with distilled water to a volume of 200 μl. The untreated group and the DNFB group were treated with the same volume of distilled water. Sucrose and aspartame were given orally every day from days 9 to 17.

Allergen sensitization and challenge

DNFB sensitization was induced by the repeated application of 25 μl of 0.2% DNFB in acetone/olive oil (3:1) to the inner and outer surfaces of the right ear, and 100 μl of the same solution was applied to shaved dorsal skin once on days 3 and 6. On days 9, 12, and 15, sensitized mice were challenged with 0.3% DNFB to the dorsal and right ear skin surfaces. Control mice were treated with the same volume of vehicle. Increasing ear thicknesses were measured by a Digimatic Indicator thickness gauge (Mitsutoyo, Tokyo, Japan).

Dermatitis score

The severity of AD–like clinical manifestations was macroscopically evaluated by the SCORAD (SCORing Atopic Dermatitis) method (Oranje *et al.*, 2007). The degree of each symptom was graded from 0 to 3 (0, absence; 1, mild; 2, moderate; 3, severe). This scoring was based on the severity of edema, erythema, oozing, crust, excoriation, and lichenification. Overall dermatitis score was determined from the sum of all individual scores. Assessment was performed by an investigator who was blind to the grouping of the animals.

Measurement of cytokine production

T lymphocytes were isolated from draining lymph nodes of mice, and $CD4^+$ T cells were purified using a Biomag separation column (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated $CD4^+$ T cells (1×10^6) were cultured in 24-well flat-bottom culture plates in RPMI-1640 medium with 50 μM β-mercaptoethanol and 10% heat-inactivated fetal bovine serum, and then stimulated with 5 μg ml^{-1} immobilized anti-CD3 and 2 μg ml^{-1} soluble anti-CD28 antibody for 72 h at 37 °C in a 5% CO_2 atmosphere. After incubation, culture supernatants were collected and the production of IL-4 and IFN-γ was quantified by ELISA (Biolegend, San Diego, CA).

Measurement of total and DNP-specific IgE

On day 18, total serum samples were prepared and quantified using OptEIA Set Mouse IgE ELISA Kits (BD Pharmingen, San Diego, CA) and mouse anti-DNP IgE ELIAS Kits (Alpha Diagnostic International, San Antonio, TX) according to the manufacturer's instructions. The absorbance was immediately read at 450 nm on an EL 800 ELISA Reader (Bio-Tek, Winooski, VT).

Histological analysis

On day 18, right ears and dorsal skins were removed and fixed with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). Fixed tissues were washed in 30% sucrose solution and frozen in OCT compound. Cryostat sections (8 μm) were mounted on slides and dried overnight at room temperature before being fixed in ice-cold acetone for 5 min. Slides were rehydrated in phosphate-buffered saline and blocked with 5% normal goat serum in phosphate-buffered saline containing 0.3% Tween-20 (washing buffer) for 2 h at room temperature. After washing, sections were incubated with primary anti-CD4 antibody overnight at 4 °C, washed, and incubated with Alexa Fluor 488–conjugated goat anti-rat antibody for 6 h at 4°C. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole for 4 h at 4 °C. Sections were mounted and images were captured using an Eclipse 50i fluorescence microscope (Nikon, Tokyo, Japan). Skin sections (8 μm) from paraffin blocks were also stained with hematoxylin and eosin for inflammatory cells, and mast cells were stained using toluidine blue. Numbers of $CD4^+$ T cells, eosinophils, and mast cells were expressed as average total counts in three fields of 100 μm^2 (×200).

Statistical analyses

Data are presented as the mean ± SEM of at least three independent experiments performed in triplicate. Statistical analysis was performed using one-way analysis of variance followed by Bonferroni's multiple comparison test. When not shown, error bars lie within symbols. *P*-values of <0.05, <0.01, and <0.001 were considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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